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The N-terminal helix α_0 of hepatitis C virus NS3 protein dictates the subcellular localization and stability of NS3/NS4A complex

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ABSTRACT

The N-terminal amphipathic helix α_0 of hepatitis C virus (HCV) NS3 protein is an essential structural determinant for the protein membrane association. Here, we performed functional analysis to probe the role of this helix α_0 in the HCV life cycle. A point mutation M21P in this region that destroyed the helix formation disrupted the membrane association of NS3 protein and completely abolished HCV replication. Mechanistically the mutation did not affect either protease or helicase/NTPase activities of NS3, but significantly reduced the stability of NS3 protein. Furthermore, the membrane association and stability of NS3 protein can be restored by replacing the helix α_0 with an amphipathic helix of the HCV NS5A protein. In summary, our data demonstrated that the amphipathic helix α_0 of NS3 protein determines the proper membrane association of NS3, and this subcellular localization dictates the functional role of NS3 in the HCV life cycle.

Introduction

Hepatitis C virus (HCV) is the member of the distinct *Hepacivirus* genus in *Flaviviridae* family. Its infections are often persistent and lead to chronic hepatitis, which can develop into liver cirrhosis, and hepatocellular carcinoma. Currently, over 170 million people worldwide are infected with HCV. The standard of care is the combination therapy of interferon-*a* (IFN-*a*) and ribavirin, however, the effectiveness of this treatment is insufficient especially for the patients infected with HCV of genotype I.

HCV contains a 9.6-kb positive single-stranded RNA genome that encodes a polyprotein precursor of about 3000 amino acids flanked by 5′- and 3′-UTR regions that are important for translation and replication of the viral RNA (Moradpour et al., 2002; Takamizawa et al., 1991). The polyprotein precursor is co- and post-translationally processed by cellular and viral proteases to yield mature structural (Core, E1, E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Hijikata et al., 1991, 1993; Reed and Rice, 1998; Shimotohno et al., 1995). NS3 is a bifunctional molecule with an N-terminal serine protease domain which, together with the NS4A cofactor, is essential for cleavage at NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B sites, and a C-terminal domain functioning as an RNA helicase/NTPase (Bartenschlager et al., 1993; Eckart et al., 1993; Grakoui et al., 1993b; Kim et al., 1995). In addition, NS3 is the cofactor of NS2 cysteine autoprotease responsible for cleavage at NS2/NS3 site. Both enzyme activities have been well characterized, and high-resolution structures have been solved. The serine protease domain adopts a chymotrypsin-like fold with residues H57, D81 and S139 forming its catalytic triad (Kim et al., 1996). The NS3 helicase unwinds RNA and DNA homoduplexes and heteroduplexes in a 3′ to 5′ direction (Kim et al., 1995).

Formation of a membrane-associated replication complex, composed of viral proteins, replicating viral RNA, and altered cellular membranes, is a hallmark of all plus-strand RNA viruses including HCV. HCV RNA replication occurs in association with altered cytoplasmic membranes which are termed as “membranous web” (Egger et al., 2002; Moradpour et al., 2003). NS4B is sufficient to induce the membranous web formation and has been proposed to serve as a scaffold for replication complex assembly. NS5B, the RNA-dependent-RNA polymerase, is an essential component of this RNA replication machinery (Lohmann et al., 1997). Almost all the HCV nonstructural proteins have been studied for structural determinants of membrane association. The N-terminal domain of NS4A is predicted to form a transmembrane α-helix involved in membrane anchorage of the NS3-4A complex (Brass et al., 2008; Gosert et al., 2005). Moradpour et al. (Gouttenoire et al., 2009b) reported that an amphipathic α-helix at the C terminus of NS4B (amino acid residues 229 to 253) was essential for membrane association and was involved in the formation of a functional HCV replication complex. In addition, an amphipathic α-helix extending from amino acids 5 to 25 of the N-terminus of NS5A was identified as a membrane anchor domain by NMR spectroscopy (Penin et al., 2004). For NS3, the amphipathic

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helix α_0, formed by the N-terminal amino acid residues 12–23 highly conserved among HCV genotypes, was reported to serve as an essential structural determinant for the membrane association of the NS3-NS4A complex. However, the detailed virological and biological functions of this amphipathic helix in the HCV life cycle have not been fully explored.

Here we analyzed the detailed functions of the amphipathic helix α_0 of NS3 in the HCV life cycle. We demonstrated that the helix α_0 served as the primary structural determinant for the membrane association of NS3 and NS3/4A protease complex, and this subcellular localization prevented the degradation of the NS3 protein.

Results

M21P mutation destroyed the N-terminal amphipathic α helix of NS3 protein

A previous study demonstrated that the N-terminal 10–24 residues of HCV NS3 protein formed an amphipathic helix α_0, a critical determinant for ER membrane association of NS3-NS4A complex (Brass et al., 2008). However, the biological function of this α helix in HCV life cycle has not been fully explored. We engineered a point mutation (M21P) from methionine to proline at amino acid 21 of NS3 of JFH1, a genotype 2a HCV strain (Fig. 1A). Secondary structure prediction analysis indicated that the M21P mutation impaired the helix structure formation, as the predicted overall 22% helix content for the wild-type decreased to 11% for the mutant peptide (Fig. 1B). This prediction was further confirmed by circular dichroism analysis of the peptides covering the N-terminal 10–24 amino acids of JFH1 NS3 protein. As shown in Fig. 1C, the wild-type peptide displayed a typical alpha helix absorption curve, while the M21P peptide did not. These results demonstrated that the M21P mutation destroyed the N-terminal amphipathic alpha helix of NS3.

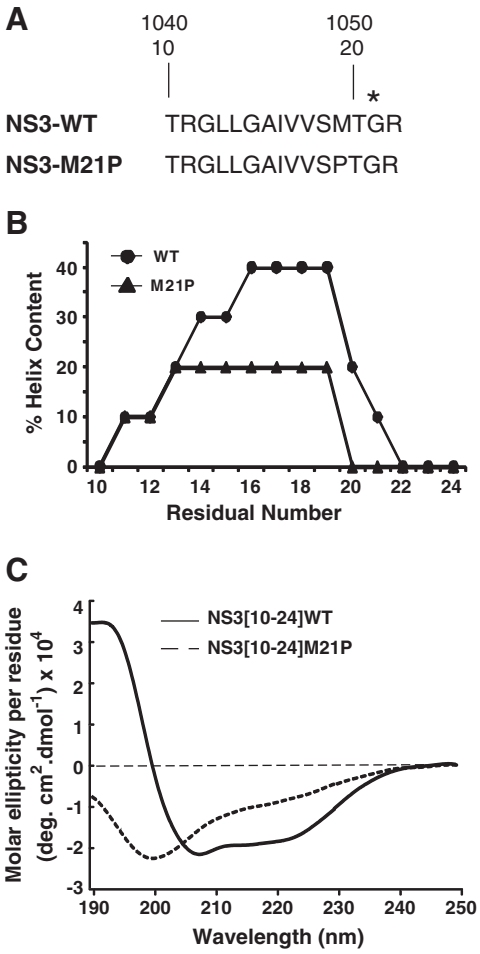

Fig. 1. M21P destroyed the N-terminal amphipathic helix α_0 of NS3. A. Schematic representation of amino acids 10–24 of wild-type and M21P mutant NS3 protein (JFH1 strain). The point mutation at position 21 is indicated by the asterisk. Nucleotides (1040–1050) and amino acids (10–20) are numbered according to the HCV polyprotein and NS3 respectively. B. Prediction of the secondary structure of amino acids 10–24 segment of NS3 using Agadir software (http://agadir.crg.es/) (Munoz and Serrano, 1994) for the helix contents. C. Circular dichroism analysis of peptides encompassing the amino acids 10–24 of wild-type and M21P mutant NS3 proteins.

Disruption of the helix α_0 did not affect NS3 helicase/NTPase activities

HCV NS3 protein is a multifunctional protein with helicase and NTPase activities in the C-terminal two-thirds. Therefore, we examined whether disruption of α_0 at the N-terminus affected the helicase/NTP activities. We expressed and purified His-tagged wild-type and M21P mutant recombinant NS3 proteins. All recombinant NS3 proteins were purified to greater than 95% purity (Fig. 2A). Next we examined the helicase and NTPase activities of recombinant NS3 proteins using the previously reported assays (Zhang et al., 2005). For the helicase assay, a synthetic 30-nucleotide 5′-end ^{32}P-radiolabeled oligonucleotide was annealed to a 54-nucleotide single-stranded DNA to form a partially duplex DNA substrate with 5′ and 3′ overhanging ends. Upon unwinding by NS3 helicase, the 30-nucleotide labeled DNA strand was released and trapped by a complementary capture strand to form a duplex. As shown in Fig. 2B, both the wild-type and M21P NS3 proteins efficiently unwound the DNA duplex substrate in a dose-dependent manner. Similarly, for the NTPase assay, the wild-type and M21P NS3 proteins hydrolyzed γ-^{32}P GTP with comparable efficiencies (Fig. 2C). These results demonstrated that the M21P mutation did not affect the helicase or NTPase activities of NS3.

Disruption of the helix α_0 abolished HCV replication

To determine the functional role of the NS3 helix α_0 in HCV life cycle, the M21P mutation was introduced into pSGR-luc-JFH1, a bi-cistronic subgenomic replicon that expressed firefly luciferase, for direct detection of luciferase as a measurement of HCV RNA replication capacity (Targett-Adams and McLauchlan, 2005). Wild-type, M21P mutant and NS5B polymerase defective GND mutant (Wakita et al., 2005) *in vitro* transcribed replicon RNAs were electroporated into Huh7 cells, and luciferase activities were measured at 4, 24 and 48 h post-electroporation. As shown in Fig. 3A, the luciferase activity of the wild-type replicon increased about 30-fold after 48 h of electroporation, whereas the luciferase activity of the M21P and GND replicons declined below the basal levels after 4 h, indicating the M21P mutation abolished the HCV RNA replication.

Next, we used the infectious HCVcc system (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) to examine the effect of the M21P mutation on HCV life cycle. Wild-type and M21P mutant JFH1 genomic RNAs were electroporated into Huh7.5.1 cells. Consistent with the results of subgenomic replicon transfection, M21P mutant JFH1 RNA did not produce infectious viruses, whereas wild type displayed viral expansion kinetics as previously described (Zhong et al., 2005), with extracellular infectivity titers peaking at 1500 ffu/ml on day 21 post-electroporation (Fig. 3B). Furthermore, immunofluorescent staining of HCV E2 proteins indicated that cells transfected with M21P mutant remained negative throughout the entire experiment (Fig. 3C), strongly suggesting that the M21P mutation completely abolished HCV replication.

Next, we examined the effects of the M21P mutation on HCV non-structural protein expression. Plasmids that contain wild-type, M21P, a NS3 protease-defective mutant H57A (Bartenschlager et al.,